

Effects of ozone therapy in an experimental murine model of *Candida albicans* vulvovaginitis

Efeitos da ozonioterapia em modelo experimental murino de vulvovaginite por *Candida albicans*

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Abstract

The antifungals used in the treatment of vulvovaginal candidiasis, although effective, can have side effects and high cost, which may not occur with the medicinal use of ozone. The study aimed to evaluate the effects of medicinal home ozone therapy on *C. albicans*. The experiment was carried out in vitro, cultivating *C. albicans* in 10mm Petri dishes, containing solid Agar Sabour and Dextrose (Oxoid). The in vivo experiment used 45-day-old female C57/BL6 mice and after suppression of ovarian activity, 20 µL of *C. albicans* suspension in sterile PBS, at a concentration of 10⁵ yeast cells, were inoculated in a single dose under anesthesia. Distribution occurred in 4 groups: (i) Absolute Control Group (GCA): Not subjected to infection by the pathogen. (ii) Control Group (CG): Submitted to *C. albicans* infection, but not treated. (iii) Ozone Therapy Group (GO): Subjected to infection and treated with medicinal ozone therapy. (iv) Lavage Group (GL): Subjected to infection and subsequently treated with vaginal lavage. It was found that GC had a higher amount of CFU when compared to GO. Exposure for 600s was able to reduce the number of CFUs by 98.89% when compared to GC. The study concludes that ozone gas showed great efficacy in the proliferation of *C. albicans* in vitro. The best results were observed with application in liquid medium, however, ozone therapy was able to significantly reduce infection in the experimental model used. In in vivo experiments, the number of CFU/ml found was lower in the ozone-treated group.

Keywords: *Candida Albicans*; Candidiasis; Ozone; Murinae; Teaching.

Resumo

Os antifúngicos utilizados no tratamento da candidíase vulvovaginal embora eficazes, podem ter efeitos colaterais e alto custo, o que pode não ocorrer com o uso medicinal do ozônio. O estudo objetivou avaliar os efeitos da ozonioterapia doméstica medicinal sobre *C. albicans*. O experimento foi conduzido in vitro, cultivando *C. albicans* em placas de Petri de 10mm, contendo meio sólido Agar Sabour e Dextrose (Oxoid). O experimento in vivo utilizou

camundongos C57/BL6 fêmeas de 45 dias e após supressão da atividade ovariana, 20 µL de suspensão de *C. albicans* em PBS estéril, na concentração de 105 células de levedura, foram inoculados em dose única, sob anestesia. A distribuição ocorreu em 4 grupos: (i) Grupo Controle Absoluto (GCA): Não submetido à infecção pelo patógeno. (ii) Grupo Controle (GC): Submetido à infecção por *C. albicans*, mas não tratado. (iii) Grupo Ozonioterapia (GO): Submetido a infecção e tratado com ozonioterapia medicinal. (iv) Grupo Lavagem (GL): Submetido à infecção e posteriormente tratado com lavagem vaginal. Verificou-se que GC apresentou maior quantidade de UFC quando comparada com GO. A exposição por 600s foi capaz de reduzir em 98,89% o número de UFCs quando comparado ao GC. O estudo conclui que o gás ozônio mostrou grande eficácia na proliferação de *C. albicans* in vitro. Os melhores resultados foram observados com aplicação em meio líquido, no entanto, a ozonioterapia foi capaz de reduzir significativamente a infecção no modelo experimental utilizado. Nos experimentos realizados in vivo, o número de UFC/ml encontrado foi menor no grupo tratado com ozônio.

Palavras-chave: Candida Albicans; Candidíase; Ozônio; Murinae; Ensino.

Resumen

Los anti fúngicos utilizados en el tratamiento de la candidiasis vulvovaginal, aunque efectivos, pueden tener efectos secundarios y de alto costo, que pueden no ocurrir con el uso medicinal del ozono. El estudio tuvo como objetivo evaluar los efectos de la ozonoterapia medicinal en el hogar en *C. albicans*. El experimento se realizó in vitro, cultivando *C. albicans* en cajas de Petri de 10 mm, conteniendo Agar Sabour sólido y Dextrosa (Oxoid). El experimento in vivo utilizó ratones hembra C57/BL6 de 45 días de edad y después de la supresión de la actividad ovárica, se inocularon 20 µL de suspensión de *C. albicans* en PBS estéril, a una concentración de 105 células de levadura, en una dosis única bajo anestesia. La distribución se produjo en 4 grupos: (i) Grupo Control Absoluto (GCA): No sometido a infección por el patógeno. (ii) Grupo de Control (GC): Sometido a infección por *C. albicans*, pero no tratado. (iii) Grupo de Ozonoterapia (GO): Sometidos a infección y tratados con ozonoterapia medicinal. (iv) Grupo de lavado (GL): Sometido a infección y posteriormente tratado con lavado vaginal. Se encontró que GC tenía una mayor cantidad de UFC en comparación con GO. La exposición durante 600s pudo reducir el número de UFC en un 98,89 % en comparación con GC. El estudio concluye que el gas ozono mostró una gran eficacia en la proliferación de *C. albicans* in vitro. Los mejores resultados se observaron con la aplicación en medio líquido, sin embargo, la ozonoterapia logró reducir significativamente la infección en el modelo experimental utilizado. En experimentos in vivo, el número de UFC/ml encontrado fue menor en el grupo tratado con ozono.

Palabras clave: Candida Albicans; Candidiasis; Ozono; Murinae; Enseñanza.

1. Introduction

Candida albicans is a species of fungus only found in 80% of the human population, often dwelling the digestive system (Naglik JR et al., 2003). It has the ability to change its saprophytic, unicellular morphology, forming hyphal spores according to environmental conditions (Banting & Hill, 2001).

The *C. albicans* is part of the normal flora of healthy individuals and do not produce harmful health effects, however, if the immune system of the host is compromised, the microorganism tends to exhibit aggressive manifestations, may cause oral or intestinal Vulvovaginal Candidiasis (Naglik JR et al., 2003). It is estimated that at least 55% of all women will have at least one episode of vulvovaginitis *C. albicans* during life (Foxman et al., 2000). Other estimates indicate that 1 in 4 women seen in a gynecology outpatient clinic in one year had asymptomatic vulvovaginal candidiasis and 60% of them had symptoms (Ribeiro et al., 2001).

Some factors such as the use of antibiotics, hormonal contraceptives, corticoids, diseases such as diabetes or the use of synthetic underwear can alter the normal vaginal flora and defense mechanisms, favoring infection by the pathogen. The infections also happen in patients with severely compromised immune system, such as in HIV carriers as a result of anti-neoplastic or immunosuppressive therapy after organ transplantation (Álvares et al., 2007; Colombo & Guimaraes, 2003). Among the clinical manifestations of vulvovaginal candidiasis, itching, leucorrhoea, whitish plaques, edema and erythema in the vulva and vagina can be observed (Boatto et al., 2006).

The treatment of candidiasis consists of antifungals in the form of pills or ointments applied directly to the affected area (Ferrazza et al., 2005). Although they have great efficacy, antibiotics may have high cost and undesirable side effects,

such as gastrointestinal disturbances, fever, chills, thrombophlebitis, liver toxicity, neurotoxicity, allergic reactions and / or cardiac arrest (during rapid infusion of the drug) (Castro et al., 2006).

A simple low cost proposal, of easy access and with few side effects for the treatment against protozoa, bacteria, viruses, and including fungi, is based not on the use of the medical ozone (Ferreira et al, 2013; talukdar et al., 2015).

In 1834, ozone (O₃) was found by German chemist Friedrich Cristian Schönbein and currently, after 125 years of research and applications, its use as a therapeutic agent is recognized worldwide. In Brazil, ozone therapy began to be used around 1970 by physician Heinz Konrad. O₃ promotes the formation of Species Reactive oxygen molecules (SRO) which influence in cellular metabolism, providing benefits for wound healing, and antimicrobial effects, antibacterial and fungicide (Oliveira, 2011). When in contact with injured tissue, ozone promotes the generation of SRO as hydrogen peroxide, increasing glycolysis, forming adenosine triphosphate (ATP) and accelerating the transport of oxygen to the tissues. Thus, it favors the response of the immune system, as it activates leukocytes and increases the production of interleukins and cytokines. Tissue repair actually occurs with increased platelet activity and increased release of growth factors. The formation of granulation tissue and neovascularization occurs, which accelerates tissue healing (Traina, 2008; Martínez-Sánchez et al., 2012).

The ozone therapy is a technique that uses the O₃ as a therapeutic agent and has been applied in situations of osteomyelitis, abscesses, decubitus ulcers, diabetic foot, burns, ischemic diseases, pulmonary diseases, among others. The effects of ozone therapy include the formation of granulation tissue and neovascularization due to its septic properties, stimulation of blood circulation, revitalization of organic functions and activation of the body's immune system (Bearzatto et al., 2003; Bocci, 2005).

The use of medical ozone for the treatment of wounds have shown good results, both in the total wound healing as in the stimulation and improvement of the healing process, improving the appearance and the smell. Tests stated that, due to the improvement in the wound healing process, the need for amputation in several cases was exempted (Oliveira, 2007; Wainstein et al., 2011).

This work aims to evaluate the effects of medicinal ozone therapy on *C. albicans* through *in vitro* and *in vivo* assays using the murine experimental model of vulvovaginitis. For this, the vulvovaginal histopathological changes caused by the fungus *C. Albicans* will be evaluated in vulvovaginal candidiasis in C57/BL6 mice by counting the Colony Forming Units before and after the collection of the vaginal wash. Finally, the effects obtained with the application of ozone therapy in relation to the control group will be discussed.

2. Methodology

2.1 *In vitro* experiment

2.1.1 Cultivation of strains and adjustment of concentration

Strains of *Candida albicans* were grown for 24 h at 37 °C on solid medium Sabouraud Dextrose Agar (Oxoid). Then, part of the cell mass of each strain was removed, for further dilution in distilled water and the concentration adjusted to level 2 of the Mc Farland Scale (PhoenixSpec Calibrator).

2.1.2 Control board making

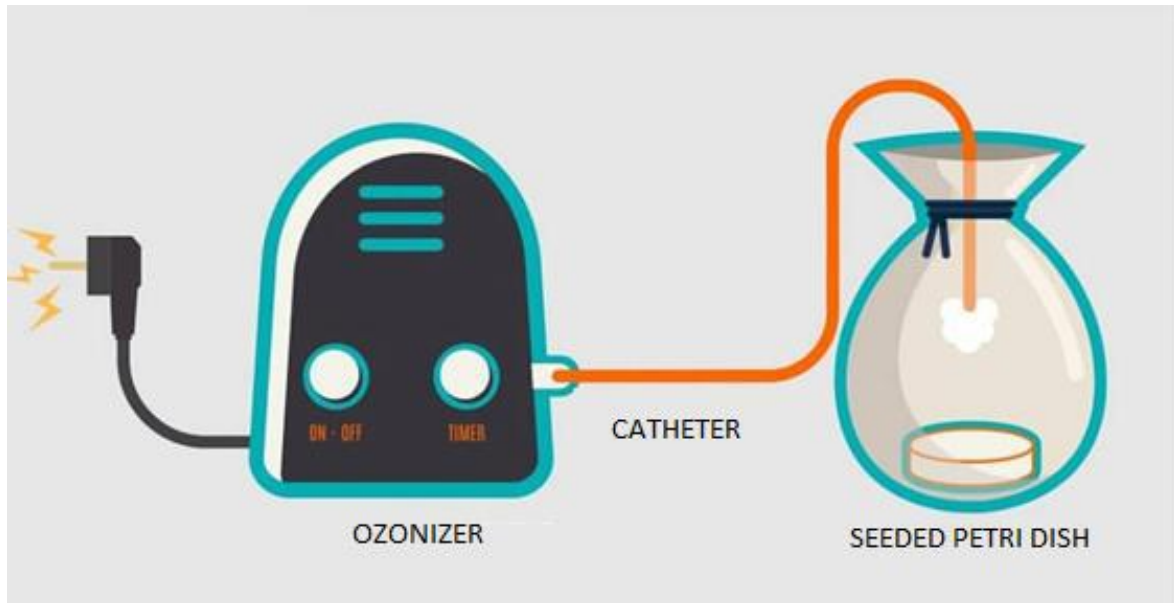
In the plates used for the control, 10 µL of the cell suspension of *C. albicans* were also seeded, with a calibrated loop, in 10 mm Petri dishes, containing solid medium Agar Sabourand Dextrose (Oxoid). These plates have not been subjected to ozone exposure.

2.1.3 Sowing of suspensions and exposure in an ozone camera

Obtaining the dilution of each strain, 10 μL of each cell suspension were seeded, with a calibrated loop, in 10 mm Petri dishes, containing solid medium Agar Sabourand Dextrose (Oxoid). The plates were immediately placed inside a transparent plastic bag with the lids open, and submitted to the release of ozone by the Vigor domestic generator 400mg/h at a concentration of 1.8 g/m^3 .

The plates were exposed inside the ozone chamber for different periods: 30 s, 60 s, 150 s and 600 s. (figure 1).

Figure 1. Scheme of the exposure of plates with *C. albicans* to a domestic ozonizer with a sealed plastic bag.



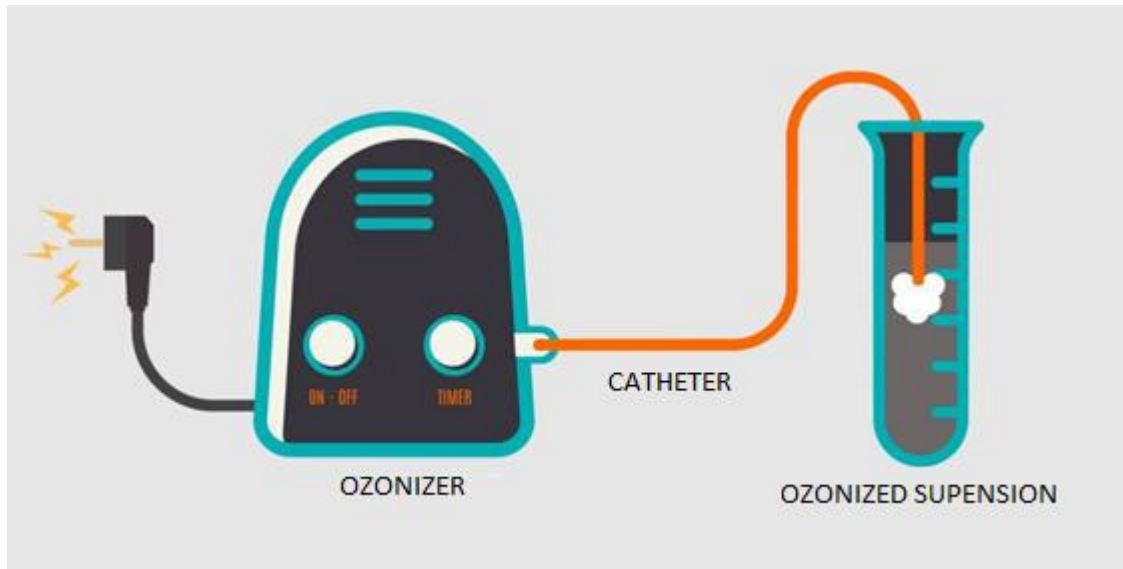
Source: Authors.

2.1.4 Sowing and ozonation of suspensions

In addition to exposure to ozone through the air, the strains were also exposed in a liquid medium. Immediately after adjusting the concentration of the *C. albicans* cell suspension to *McFarland* index 2, the *C. albicans* suspensions were ozonized.

In a test tube containing the cell suspension, the suspension of *C. albicans* was ozonated using a thin-caliber nasogastric cannula (Figure 2). Ozonation took place for 30 s and 60 s. Then, the exposed suspensions were plated using 10 microliters of each cell suspension, with a calibrated loop, in 10 mm Petri dishes, containing solid medium Agar Sabourand Dextrose (Oxoid).

Figure 2. Schematic of *C. albicans* solution to the domestic ozonizer in a test tube.



Source: Authors.

2.1.5 Cultivation of the plates and counting the CFUs

After plating, the plates were stored in an oven at 36 °C until the time of counting. The number of Colony Forming Units was obtained between 24 and 48 h after plating. All tests were done in triplicate and repeated 3 times. The number of CFU obtained on each plate was compared to the number of CFU observed on the control plate.

2.2 *In vivo* Experiment

As for the animals used, were mice C57 / BL6, females, with 45 days of life, from the Central Animal Laboratory of Western Paraná State University (UNIOESTE). The animals received a standard diet of pellets and water *ad libidum*, were kept in standard crates in groups of 3 animals, light and dark cycle of 12 hours distributed in four distinct groups, each formed by 6 animals.

2.2.1 Development of the experimental model of vulvovaginitis

First the ovarian activity of the female was suppressed using Estradiol Valerate orally (by gavage) diluted in 0.2 ml of distilled water at a dose of 2mg / kg / day for 7 days. After suppression of ovarian activity, 20 µL of *Candida albicans* suspension in sterile PBS was inoculated, in a single dose and under anesthesia, at a concentration of 10⁵ yeast form cells according to YANO et al., (2011). Females from all groups in the experiment described below, except for the control group, were submitted to model development (Figure 3).

Figure 3. Inoculation of *C. albicans* suspension



Source: Yano et al. (2011).

2.2.2 Distribution of groups

The distribution was given in 4 groups of animals: I Absolute Control Group (ACG): Not submitted to infection by the pathogen. Used to control amongst all groups. II Control Group (CG): Subjected to *C. albicans* infection, but not treated. Used for comparison amongst treated groups. III Group ozone therapy (GO): Subjected to pathogen infection and treated with ozone therapy of medical use. IV Washing Group (WG): Submitted to infection and later treated with vaginal wash. Flagged as control group for GO.

2.2.3 Proof of infection

Following the methodological processes of microbial culture, the collection and plating of the vaginal wash of the females was carried out. For this purpose, sterile saline solution was introduced (20 μ L) into the vaginal tube with micropipettor then, immediately removed and seeded in Petri dishes containing medium ASD-chloramphenicol. The solution was incubated at 37 °C for 48-72 h. Infections were considered positive if there was growth of at least one yeast colony in individual counting and an average score of all cultures from each animals was 10 CFU / m L (Marcia *et. Al.*, 2010). The same procedure was performed in GCA mice.

2.2.4 Treatments

For the Group Ozone therapy was performed the procedure of vulvovaginal washing once a day for three days. The procedure took place with 10 m L of distilled water previously ozonated, for immediately vaginal instillation with the use of a neonatal probe, number 4. The probe tip has been previously greased with Xylocaine gel 2% and inserted carefully into the animal's vagina, for slow instillation of 10 mL of ozonated water, using a 10 mL syringe. In the Washing Group, the same procedure was performed, but with distilled water, not ozonated.

2.2.5 Collection of vaginal washing and sacrifice

Promptly before sacrificing the rodents, it was performed the procedure of collecting of the vaginal wash. 50 μ L of sterile PBS was instilled into the vagina of each animal, using a micropipettor and 100 μ L tip, and aspirated immediately afterwards.

Each wash was plated in a Petri dish and all dishes were kept in an oven at 35 °C for counting CFU after 24 h.

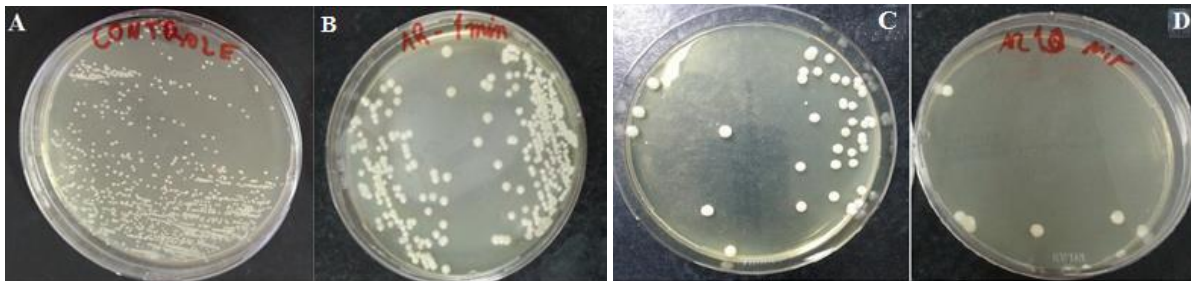
After collecting the vaginal aspirate, the animals were anesthetized with 50 mg/kg of Ketamine and 10 mg/kg of Xylazine intraperitoneally sacrificed by decapitation in a guillotine.

The data obtained from the CFU were analyzed through descriptive and analytical statistics using the ANOVA test, using the Tukey test *as post hoc*, with alpha set at $p > 0.05$.

3. Results

Figure 4 shows the images of the Petri dishes after 24 h for the *in vitro* experiment and exposure to ozone in the chamber. The control plate had a greater amount of CFU (Figure 4A) when compared to plates exposed to ozone by air (Figures 4B, C and D). Exposure for 600 s proved able to reduce the number of 98.89% UFC when compared to the plaques control. The ANOVA test showed a statistically significant difference among the means of CFU on the plates. The Tukey test showed that there was a difference between the means of the plates exposed for 60 s or more, when compared to the control plates or plates exposed for 30 s ($p = 0.0001$) (Table 1).

Figure 4. In vitro assays. Petri dishes after 24 h of exposure to ozone. A): Not treated with ozone; B): exposed to ozone for 60 s; C): exposed to ozone for 150 s; D): Exposed to ozone for 600 s.



Source: Authors.

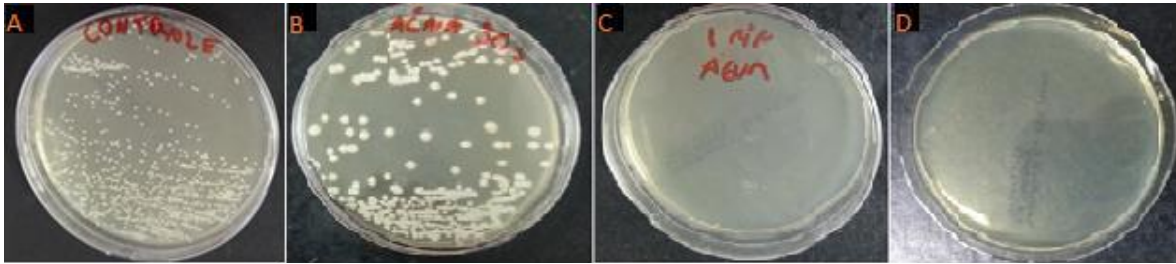
Table 1. Averages of CFU/ml of the plates exposed to ozone. Percentage of cell growth rate and percentage of difference among the average number obtained.

Exposure time by air	Average CFU/ml	Growth rate (%)	Difference (%)
Control (not exposed)	720	100,00	-
60 s	160	22,22	- 77,78
150 s	36	5,00	- 95,00
600 s	8	1,11	- 98,89

Source: Authors.

As for the experiment with ozonated suspensions, Figure 5 shows the images of the Petri dishes after 24 h for the *in vitro* experiment. It is noted that suspensions ozonated for 60 s (figure 5C) and 150 s (figure 5D) did not show cell growth, obtaining a difference of 100% when compared to the control plate (figure 5A). The amount of UFCs counted in the ozonated suspensions are shown in Table 2.

Figure 5. Tests in vitro. Petri dishes 24h after sowing the ozonated suspensions. figure 5A: Control; figure 5B: Treated for 30 seconds; figure 5C: treated for 60 seconds; figure 5D: treated for 150 seconds.



Source: Authors.

Table 2 - Presentation of average CFU/ml of the ozonated suspensions, percentage of cell growth rate and difference among the averages obtained.

Suspension ionization time	Average of CFU/ml	Crowth (%)	Difference (%)
Control (not exposed)	720	100,00	-
30 s	110	15.27	84,73
150 s	0	0	100,00

Source: Authors.

The ANOVA test showed a statistically significant difference among the averages of UFC on the plates. The Tukey test showed that there was a difference among all comparisons ($p=0.0001$).

In experiments conducted *in vivo*, the number of CFU /ml found was lower in the group treated with ozone. The results are shown in Table 3.

Table 3 - Presentation of the CFU/ml averages of vaginal washing of the groups of females in the experiment, percentage of cell growth rate and percentage of difference among the averages obtained.

Groups	Average of CFU/ml	Growth rate (%)	Difference (%)
GC	356	100,00	-
GL	234	65.70	34.30
GO	49	13.76	86.24
GCA	0	0	100,00

Source: Authors.

The ANOVA test showed a statistically significant difference among the CFU average of the groups. The Tukey test showed that there was a difference among all comparisons ($p=0.0001$).

4. Discussion

For the first time, a study was carried out on the use of ozone therapy on the experimental model of vulvovaginitis.

A study by Cardoso et al. (2009), evaluated the effects of ozone therapy at treating wounds standing diabetics. The treatment consisted on baths with ozonated water, followed by dressings with ozonated oil. After 14 weeks of treatment, the authors reported complete wound healing. Traina (2008), also obtained similar results with ozone therapy on dermal wounds of

rats. Their results showed a reduction on the wound area, favoring tissue formation and absence of toxicity. Similarly, the results of the current study were positive in the treatment of vulvovaginitis used in the model with the application of ozone therapy, pointed out by the 86% reduction in the number of CFU in the vaginal wash of females in the treated group.

Noites et al. (2014), compared the effect of gaseous ozone with sodium hypochlorite and chlorhexidine on the microorganisms *E. faecalis* and *C. albicans*. In this study, isolated ozone, applied in short periods of time (24 and 60 s), did not show efficacy in any of the antimicrobial agents tested. However, the results indicated that with the application of 2% chlorhexidine associated with exposure to gaseous ozone, during 24 s, there was the complete elimination of *C. albicans* and *E. faecalis*. Furthermore, exposure to gaseous ozone for 180 s was significantly more effective eliminating *C. albicans* when compared to shorter times. Similar results were found at the present study, and exposure of *C. albicans* to gaseous ozone for longer periods (150 s and 600 s) were more effective eliminating the microorganism when compared to exposure for 30 s or 60 s.

In 2007, Estrela et al., compared the effects of gaseous ozone, ozonated water and sodium hypochlorite on antimicrobial efficacy against *Enterococcus faecalis* and did not find beneficial effects for the inactivation of the bacteria in question.

Gracer and Bocci (2005) defined that ozone application in the sores area promotes cell proliferation, extracellular matrix synthesis, normalizes metabolism, increases the amount of leukocytes at the area and release growth factors, which facilitates the local flow, stimulates tissue repair and healing.

An *in vitro* experimental study compared the effects of compressed air, carbon dioxide (CO₂), helium (He) and O₃ on bacterial growth. It was observed that there was no growth in 100% of the strains exposed to ozone, unlike the other gases, which did not influence bacterial growth (Pereira *et. al.*, 2005).

Similar results were obtained in this study, because the ozone therapy proved to be able to reduce significantly the number CFU of the microorganism in question in both tests *in vitro*, and *in vivo*. Only 150 s of cell suspension exposure was able to completely inhibit CFU growth.

As for side effects, Oliveira (2007) observed that a small percentage of individuals undergoing treatment with ozone presented eczema, however, with a reduction in the concentration of the O₃ dose, the symptoms quickly disappeared. Reports of other adverse effects caused by exposure to ozone were not found in the literature.

The current study demonstrated the effectiveness of ozone therapy in the experimental model used, so that further studies are promising in order to establish this modality as an auxiliary therapy in the treatment of vulvovaginitis.

5. Conclusion

The present work evaluated the effects of medicinal home ozone therapy in *C. albicans* through *in vitro* and *in vivo* assays using the murine experimental model of vulvovaginitis. The study concludes that ozone gas showed great efficacy on the proliferation of *C. albicans in vitro*. The best results observed were the ones with application in liquid medium. Nevertheless, ozone therapy was able to significantly reduce the infection in the experimental model used. No tests were performed to observe the vaginal bacterial microbiota; new studies are suggested to observe the viability when the vaginal bacterial flora is not destroyed.

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